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MMP-9 directed shRNAs as relevant inhibitors of matrix metalloproteinase 9 activity and signaling*

MMP-9 shRNA jako inhibitory aktywności i szlaku sygnalizacji metaloproteinazy 9*

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: The main function of matrix metalloproteinases is the degradation of extracellular matrix components, which is related to changes in the proliferation of cells, their differentiation, motility, and death. MMPs play an important role in physiological processes such as embryogenesis, angiogenesis and tissue remodeling. The increase of MMPs activity is also observed in pathological conditions including tumorigenesis where MMP-2 (gelatinase A) and MMP-9 (gelatinase B) show the ability to degrade the basement membrane of vessels and they are involved in metastasis. The aim of our study was to verify the changes of MMP-9 enzymatic activity and the mobility of cells after inhibition of *MMP-9* gene expression.

Material and Methods: The oligonucleotide shRNA insert had been designed to silence *MMP-9* gene expression and was cloned into the pSUPER.neo expression vector. The construct was introduced into the HeLa (CCL-2) cervical cancer cells by lipotransfection. Simultaneously in control cells MMP-9 were inhibited by doxycycline. Changes in activity of MMP-9 were analyzed by gelatin zymography and wound-healing assay.

Results/ Conclusions: Gelatin zymography allowed us to confirm that activity of MMP-9 in cells transfected by shRNA-MMP-9 and treated by doxycycline were similar and significantly lower in comparison with control cells. Phenotypic tests of migration *in vitro* confirm statistically significant ($P<0.05$) changes in cell migration – control cells healed 3 to 5 times faster in comparison with transfected or doxycycline treated cells. Our studies show the significant role of MMP-9 in mobility and invasiveness of tumor cells, thus indicating a potential target point of interest for gene therapy.

Keywords: **Extracellular matrix metalloproteinases • metastasis of tumors • migration of cells • proteolytic activity • RNA interference • cervical cancer • zymography • wound-healing assay**

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Abbreviations: **ECM** – extracellular matrix, **MMPs** – matrix metalloproteinases, **RNAi** – RNA interference, **shRNA** – short hairpin RNA, **TIMPs** – tissue inhibitors of metalloproteinases.

Introduction

Extracellular matrix metalloproteinases (MMPs) are zinc-dependent multidomain endopeptidases consisting of a propeptide with the 'cysteine switch' motif of PRCGXPDV, a catalytic domain with a zinc binding motif and an additional linker peptide. Some MMPs also contain a hemopexin domain and fibronectin motif. MMPs can be divided into six subclasses: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and others depending on their substrate specificity [22,39].

MMPs activity is regulated during transcription by the activation of pro-MMP zymogens and by endogenous tissue inhibitors of metalloproteinases (TIMPs) [20].

These endopeptidases are activated by autolysis or by other proteinases but they also can be activated by chemical and physical factors like aminophenylmercuric acetate (APMA), low pH, and high temperature. An important role in inhibition of MMPs activity is played by TIMP, anti-inflammatory cytokines (IFN-γ or IL-4), drugs (dexamethasone and indomethacin), α2-macroglobulin, and chelates (antibiotics, thiols), which inhibit the activation of pro-MMP. A decrease in MMPs activity is also possible to achieve by blocking expression of their genes using an RNA interference strategy [20,22].

The function of MMPs is connected with their ability to hydrolyze extracellular matrix components (e.g. fibronectin, collagen, elastin, proteoglycans) and elements of endothelial cell basement membrane (e.g. laminin or type IV collagen). This degradation results in removing physical and structural barriers which promotes cell migration and changes their function under physiological and pathological conditions [13,23]. MMPs control angiogenesis, embryogenesis or tissue remodeling and wound-healing processes, which is regulated by the balance between MMPs and TIMPs under normal conditions, but they are also active in cardiovascular and autoimmunological disorders, or Alzheimer disease. Increasing activity of MMPs is observed in carcinogenesis, which is related to angiogenesis and metastasis of cancer with visible dysregulation of MMPs and TIMPs balance [12,17].

MMP-2 (gelatinase A) and MMP-9 (gelatinase B), which cleave collagen IV and degrade basement membrane, play an important role in tumor invasion [13]. It is proved that overexpression of both gelatinases is related to the progression of gynecological cancers including endometrial cancer, cervical carcinoma and ovarian carcinoma [32]. Moreover, the hemopexin domain of matrix metalloproteinases, especially in MMP-9, is involved in cell migration, where MMPs utilize a non-proteolytic mechanism of epithelial cell migration dependent on MAPK and P13K signaling pathways [6].

It is important to investigate and analyze the expression and enzymatic activity of MMPs by measuring mRNA and protein levels with several different techniques. To analyze changes of activity and the protein level, zymography is commonly used. It is an electrophoretic technique that detects MMPs by the degradation of their preferential substrate and by their molecular weight. To identify MMP-2 (72 kDa) and MMP-9 (92 kDa) gelatin zymography is mainly used [40].

A wound-healing assay is used to study the influence of MMPs on migration of cells *in vitro*, which imitate wound-healing *in vivo*. This assay involves creating a 'wound' in a cell monolayer, and monitoring the 'scratch healing' by the rate of cell migration [2].

Considering the ability of gelatinases to degrade a variety of ECM macromolecules and facilitate cell invasion and their significant role in several critical steps of cancer progression, we investigated the activity and protein level of MMP-9 in HeLa cells after silencing the target gene expression by RNA interference (using shRNA – short hairpin RNA). As a point of reference at inhibiting MMP-9 synthesis the influence of doxycycline on cells was also analyzed in studies. Currently, doxycycline is the only clinically available MMPs' inhibitor [11].

Materials and Methods

Cell culture and transfection conditions

Human cervical cancer cell line (HeLa) CCL-2™ was purchased from American Type Culture Collection (ATCC). Cells were grown in plastic culture dishes under standard conditions at 37°C in 95% humidity and 5% CO_2 in a Hera-Cell incubator (Heraeus). The RPMI-1640 medium containing L-glutamine (PAA Laboratories GmbH) was supplemented with 10% FBS (PAA Laboratories GmbH), and 10 μg/ml gentamycin. Culture conditions were monitored by microscopic analysis using an inverted microscope (Axiovert, Zeiss). Cell concentrations and viability were determined in a Neubauer Hemocytometric Chamber using a 0.4 % solution of trypan blue in physiological saline (Sigma).

shRNA construct

To obtain the efficiently silencing genetic construction required: analyses of the gene and mRNA sequence of metal-

loproteinase 9 (NM_004994.2), design and synthesis of oligonucleotides that can be expressed as shRNA (short hairpin RNA) in cells, and their cloning into pSUPER.neo expression vector (OligoEngine). The recombinant plasmids were cloned in prokaryotic *E. coli* cells, isolated by alkaline lysis, and verified using PCR, restriction analysis and sequencing. The effectiveness of conducted down-regulation was confirmed by measuring the level of MMP-9 transcripts in cancer cells by real time RT-PCR technique in an independent study (data not shown).

Transfection conditions. Doxycycline treatment

One day before transfection the human cervical cancer (HeLa) cells were plated at a density of $1.5x10^5$ cells per well in 1000 µL of growth medium with antibiotics in 12-well tissue culture plates. The next day, the level of culture confluence was verified in an inverted microscope. Cells were transfected with shRNA-MMP-9 constructions using Lipofectamine™ transfection reagent (Invitrogen), which is a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido) ethyl]-N,N-dimethyl-1- propanaminiumtrifluoroacetate (DOSPA) and the neutral lipid dioleoylphosphatidylethanolamine (DOPE) in membrane-filtered water. Serum-supplemented medium was removed, cells were washed twice with serum free medium and 500 µL of Opti-MEM Medium (GIBCO) was added. For each transfection sample 2.5 µg of plasmid DNA (coding for shRNA) was diluted in 100 µL of Opti-MEM Medium. At the same time 6 µL of Lipofectamine solution was mixed with Opti-MEM medium to a final volume of 100 µL. Diluted DNA was combined with diluted Lipofectamine Reagent (total volume 200 µL), mixed gently and incubated for 30 minutes at room temperature. Prepared mixture was added to the cells for 6 hours (transfection time) and inactivated with serum-containing medium. Non-transfected cells were cultured as a control group. In order to verify the correctness and effectiveness of transfection plasmid pEF/myc/cyto/GFP (Invitrogen) encoding the green fluorescent protein (GFP) was used. The efficiency of lipo-transfection was evaluated by monitoring the ratio of properly transfected cells showing GFP fluorescence in relation to all cells. As a reference modulator capable of inhibiting the synthesis of MMP-9 the tetracycline antibiotic doxycycline hyclate (Sigma) was used. The incubation was performed with 10 µM antibiotic solution in serum free medium for 24 hours.

Gelatin zymography

Enzymatic activity of MMP-9 was measured by gelatin zymography in 10% SDS-polyacrylamide (Sigma) separating gels in the presence of gelatin. Transfected cells, cells treated with doxycycline and control cells were subjected to conditioning in MEM medium (PAA Laboratories GmbH) without serum and antibiotics for 24 or 48 hours. The conditioned medium from each treatment was collected and centrifuged (2 000 RPM, 5 min) to remove cells and cell debris. The clear supernatants were concentrated by centrifugation (8 000 RPM, 5 min) on VIVASPIN 500 columns (Vivascience). The amount of total protein in the marked supernatants was assessed by the Bradford assay with Bradford Reagent (Sigma) according to a manufacturer's protocol. Supernatants were incubated with an adequate volume of SDS sample loading buffer for 30 minutes at 37°C and electrophoresed separated at 4°C. Following electrophoresis the gels were rinsed three times in enzyme renaturing buffer containing Triton X-100 (30 minutes per wash at room temperature). Subsequently, the gels were incubated in developing buffer (50 mM Tris-HCl, pH=7.5; 200 mM NaCl; 5 mM $CaCl_2$; 2 µM $ZnSO_4$; 0.02% Brij-35) for 48 hours at 37°C, stained with Coomassie brilliant blue R-250 solution for 12 hours and destained in methanol, acetic acid and water (5:4:1) solution until clear bands of MMP activity were visible on the dark blue background. Gelatinase zymograms were scanned using a gel documentation system (InGeniusSynGene Bio Imaging Genesnap). The intensity of bands was quantified by comparing the peak area (raw volume) corresponding to integrated optical density (IOD) using the pixel-based densitometer program Syngene GeneTools. Digestive activity of MMPs was confirmed by presence of two bands on zymograms, slower migrating MMP-9 (94 kDa) and faster migrating MMP-2 (72 kDa) in comparison with Prestained Protein MV Marker (Fermentas).

Wound-healing assay

Changes of migration and motility of cancer cells were examined using a wound-healing assay. Similarly, transfected cells, cells treated with doxycycline and control cells were seeded in 12-well tissue culture to achieve approximately 80% confluence. Using a sterile yellow pipette tip a straight scratch simulating a wound in a monolayer was made. After scratching, the wells were gently washed with medium to remove the detached cells and the fresh growth RPMI-1640 medium containing L-glutamine was added. The monolayers were then incubated at 37°C for 48 hours. The healing and the speed of cell movement across the gap were observed. Digital documentation was made after scratching (at zero time), after 24 hours and after 48 hours with an Eclipse Ti microscope (Nikon). The effectiveness of applied modulators on cell migration and motility was estimated by the relative distance of wound closure.

Statistical analysis

Zymography results and wound-healing assay results were analyzed statistically using the STATISTICA 9 program. Statistical comparisons were carried out using ANOVA and Tukey's test for analysis of significance of difference between values. Differences were considered significant at a *P* value of less than 0.05.

Results

The expression of pEF/myc/cyto/GFP plasmid encoding green fluorescence protein was visible in HeLa cells which were transfected to verify lipotransfer propriety as shown in Figure 1.

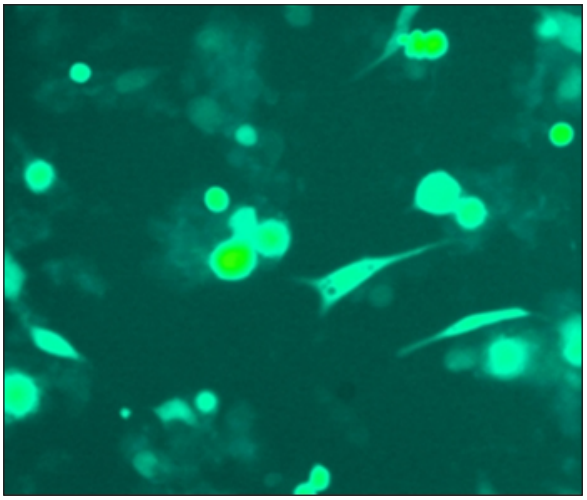

Fig. 1. Properly transfected HeLa cells where GFP protein expression was visible. Microscope Nikon Eclipse Ti, 100x

Gelatin zymography results

To investigate changes of HeLa cell phenotype caused by post-transcriptional *MMP-9* gene silencing we analyzed migration rate and capacity of cells for metalloproteinases secretion to growth medium. It was verified respectively by wound-healing assay and gelatin zymography. All differences were referred to cells not treated by interfering RNA and to doxycycline stimulated ones.

The presence of metalloproteinases was visualized by zymography technique where the amount of active MMP-9 protein in supernatants of transfected cells was compared with supernatants from control cells.

Investigated IOD (raw volume) values were proportional to the intensity of bands and corresponded to the amount and enzymatic activity of tested proteins. The Rf value was the same in all analyzed paths on both zymograms.

As shown in Figure 2 we obtained bands on zymogram of control cell supernatants and 48 hours after transfection with plasmid pSUPER.neo containing shRNA-MMP-9. Height of peak and raw volume of bands of transfected cell supernatants were considerably lower in comparison to supernatants from control cells. We did not observe any bands in the second path where supernatants from doxycycline treated cells were analyzed.

Visible bands were also obtained 72 hours after transfection and doxycycline usage in each path. The height of peak and raw volume of supernatants from control cells were the highest, and the lowest values derived from supernatants of pSUPER.neo transfected cells (Figure 3). The bands of doxycycline treated cells had slightly higher values of height of peak and raw volume in relation to transfected cells. As shown in Figure 4 the IOD value of pSUPER.neo transfected cell supernatant and doxycycline treated cell supernatant were significantly lower in comparison with control cell supernatant after 48 as well as after 72 hours.

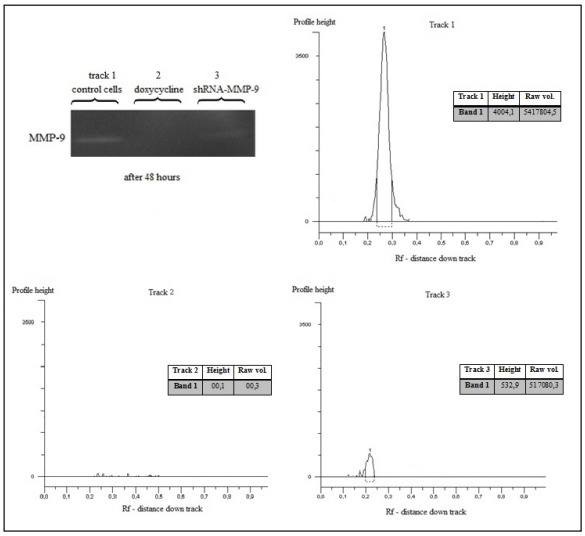

Fig. 2. MMP-9 detected in control HeLa cells and shRNA-MMP-9 transfected HeLa cells after 48 hours in gelatin zymography. Graphical and mathematical analysis of bands' IOD

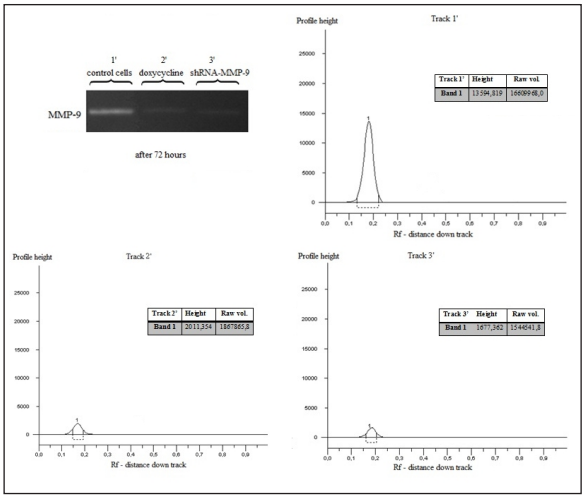

Fig. 3. MMP-9 detected in control HeLa cells, doxycycline treated HeLa cells, and shRNA-MMP-9 transfected HeLa cells after 72 hours in gelatin zymography. Graphical and mathematical analysis of bands' IOD

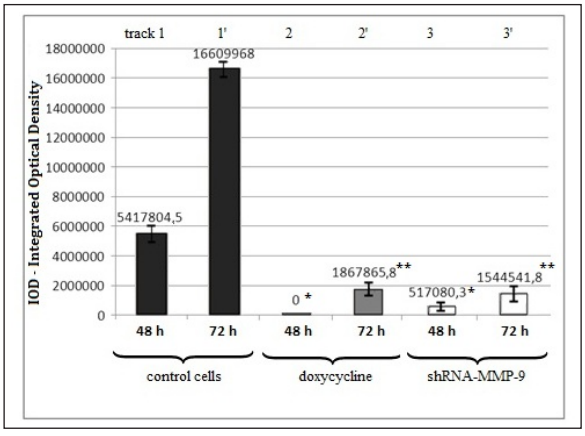

Fig. 4. Control HeLa cells' IOD values compared with shRNA-MMP-9 transfected HeLa cells' IOD and doxycycline treated HeLa cells' IOD values after 48 and 72 hours ($P<0.05$)

Wound-healing assay results

To evaluate the functional activity of the *MMP-9* expression induced by post-transcriptional silencing of this gene, we examined the cellular migration of HeLa cells using an *in vitro* wound-healing assay. This assay measures the ability of cells to migrate and fill the gap obtained by physical disruption of cell monolayers. Changes in migration and motility of adherent cells were compared in a group of cells transfected with shRNA-MMP-9 construction, cells treated with doxycycline and control cells. Representative images of this experiment are shown in Figure 5. We observed a significant delay in wound closure in both experimental groups of cells compared with control cells. The rate of wound closure was defined on the basis of the average distance between the cells located at the edges of created wounds (Figure 6). After 24 hours, the average distance between the wound edges in control cells was reduced up to six-fold. However, in shRNA--MMP-9 silenced cells and in cells treated with an antibiotic this distance was reduced only about two-fold. Whereas control cells intensively covered the wound and its complete closure was observed after 48 hours, gaps in the transfected or doxycycline treated cell remained open. To sum up, both shRNA-MMP-9 and doxycycline significantly reduced the speed of cell migration in comparison to control cells. These changes were statistically significant at the level of $P<0.05$.

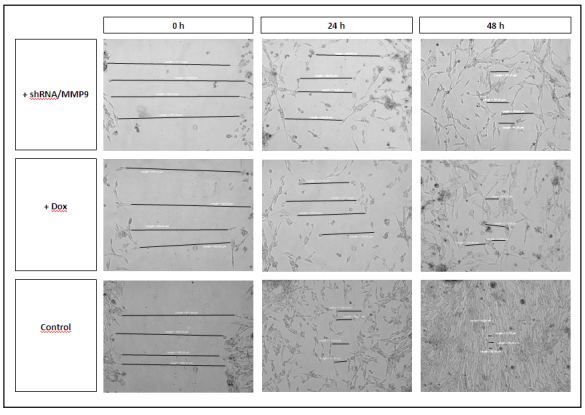

Fig. 5. Wound-healing migration assay of HeLa cells transfected with shRNA-MMP-9 or treated with doxycycline in relation to control cells. Pictures were taken at the indicated time points (0 h, 24 h, 48 h) after wounding (Microscope Nikon Eclipse Ti, 100x)

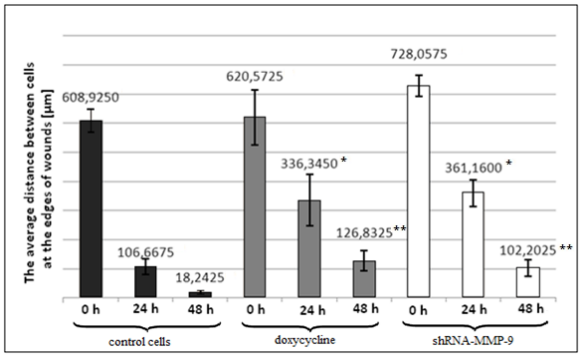

Fig. 6. The average distances between cells at the edges of the gaps in the wound-healing assay along with the standard deviation ($P<0.05$)

Discussion

The main cause of death in economically developed countries and the second leading cause of death in developing ones is cancer [14]. The process of carcinogenesis is complex and multistage. Cells of the growing tumor divide excessively, invade surrounding tissues, penetrate the blood system and form metastases in distant organs [43]. Tumor cell migration is also associated with expression of various adhesion molecules such as ICAM, VCAM and cadherins. All carcinogenic processes are directly or indirectly related to the enhanced degradation and remodeling of extracellular matrix and the degradation of basement membranes and blood vessel walls. It occurs through the activity of enzymes from a group of extracellular matrix metalloproteinases [8,15,20]. Among MMPs, in the development of cancer a crucial role is played by gelatinases, including MMP-2 and MMP-9. These enzymes are characterized by the ability to digest collagen type IV, which is the main component of basement membranes of blood vessels. Their activity affects all of the mechanisms that promote tumor growth such as cell proliferation, their adhesion, migration, angiogenesis and avoidance of a response from the immune system [13,42]. Higher levels of gelatinase secretion and activity are observed in many different cancers and they usually correlate with the severity of the disease, its aggressiveness and a shorter period of patient survival [10,22,31,37]. It has been shown that the up-regulation of MMPs is also associated with the progression of gynecological cancers including uterine cervical carcinoma [21,32]. According to 2008 data cervical cancer is the third most commonly diagnosed cancer among women, with almost 530,000 new cases each year, and the fourth leading cause of cancer death, with more than 275,000 deaths in females worldwide [14,34]. It is considered that the main cause of uterine cervical neoplasm formation is persistent infection with oncogenic types of human papillomavirus (HPV) [21,25]. The development of cervical cancer takes a long time and starts with a precancerous stage called cervical intraepithelial neoplasia. Cancer screening using the pap smear can identify the phase of dysplasia, which does not harm the health of the patient and is fully treatable. However, once the cancer spreads through metastasis, patient outcome is poor [29,30]. Therefore the studies reported in this paper were based on an established HeLa cell line representing an experimental *in vitro* model of cervical cancer.

Currently, conventional cancer therapy involves operational excision of the tumor, radiotherapy and systemic therapy involving the administration of cytotoxic drugs. However, regardless of the progress made in surgery, radiotherapy and chemotherapy, cancer treatment results are still unsatisfactory [24]. Furthermore, due to high variability at the cellular and genetic level cancer cells often exhibit natural or acquired resistance to administered drugs [38]. With the rapidly growing knowledge regarding the molecular mechanisms un-

derlying the development of cancer it becomes possible to develop new therapeutic strategies. In prevention of angiogenesis, tumor growth and metastasis an important role is played by suppression of MMPs by endogenous inhibitors such as TIMPs or α_2-macroglobulin, or by synthetic ones such as tetracyclines [16,17]. There is also a possibility to affect mRNA of MMPs by ribozymes or antisense RNA. RNA interference methods are also used, where RNA-dependent gene expression is blocked by short interfering RNA (siRNA) or by short hairpin RNA (shRNA) [27,28]. In our study HeLa cells were used to assess the impact of silencing *MMP-9* gene expression in the context of tumor cell invasiveness. Our previous investigations confirmed that the designed shRNA--MMP-9 construct changed the expression of metalloproteinase 9 gene at the mRNA level in cancer cells (4.4% mRNA MMP-9 in comparison with control cells after 24 hours, and 26.2% after 10 days in comparison with control cells).

To estimate changes of enzymatic activity the zymography technique was used. The results of IOD values show that interfering RNA technique caused a 10-fold decrease of MMP-9 activity 48 hours after transfection and almost 11-fold decrease 72 hours after transfection as compared with control cells. Enzyme level was approximately 90% decreased. Interfering RNA is commonly used to inhibit gelatinase expression. Kunigal et al. [19] investigated the MMP-9 activity *in vitro* in MDAMB231 epithelial mammary gland adenocarcinoma cell line. *MMP-9* inhibition gained by an adenoviral siRNA construct caused 40% decrease of mRNA level, and zymographic analysis showed a 60% reduction of enzyme activity. Kunigal et al. [18] also studied the decrease of *MMP-9* expression by siRNA under control of cytomegalovirus *in vitro* in MDAMB231 and ZR751 human mammary gland adenocarcinoma cell lines. Zymography analysis showed an 80% decrease of enzyme activity in ZR751 cells and 90% decrease of enzyme activity in the MDAMB231 cell line.

The activity of shRNA-MMP-9 particles was verified in relation to the results obtained by inhibition of MMP-9 synthesis with doxycycline, which is a clinically approved MMP inhibitor and an authorized medicinal product [5,26]. Tetracyclines regulate the activity of MMPs directly and indirectly during zymogen activation, which is connected with zinc ion bonding ability necessary for proper enzyme function [4]. These observations were confirmed by the reverse doxycycline inhibition gained with redundancy of Zn^{2+} [7]. Doxycycline was used as a reference modulator causing decreased activity of MMP-9 and 48 hours after usage no band in the zymogram was obtained (Fig. 2) but 72 hours after antibiotic treatment the band was seen and its IOD was 9 times smaller as compared to control cells (89% decrease). Metalloproteinase 9 needs zinc ions (Zn^{2+}) for correct activity and a probable explanation of band absence 48 hours after antibiotic usage could be strong binding of doxycycline with zinc ions [3]. Probably after some time disintegration of doxycycline-zinc complexes occurred and metalloproteinase became necessary for an activation cofactor. As shown by Smith and co-authors [35], it is possible that pH changes (decrease of pH) during cell growth time lead to inability of doxycycline to inhibit MMPs. Apart from chelate zinc ions tetracyclines inhibit MMPs activity by different mechanisms such as the influence on their mRNA stability or elimination of free radical released from ECM, which activates MMPs [36]. These effects of tetracycline action can be the reason for transient differences of MMPs activity observed in our studies. Usage of doxycycline as an MMP-9 synthesis decreasing factor is widely examined. Roomi et al. [30] analyzed modulation of MMP-9 activity by doxycycline as an inhibitor. They used the DoTc2-4510 cell line and by quantity densitometry obtained results which showed dose-dependent inhibition of MMP-9 expression (10 μM of antibiotic caused 7% decrease of MMP-9 activity, 25 μM – 16%, 50 μM – 23.5% and 100 μM – 49%). Tetracycline usage shows interim influence on stability of metalloproteinase mRNA in cells by interaction with AU-reach elements (ARE) in the 3' region of mRNA [1,44]. In turn the use of interfering RNA gives continuous and better results of MMPs inhibition. These effects can be observed as a difference in cell migration after application of interfering RNA particles. To estimate changes in migration of cells a wound-healing assay was used. The biggest changes in distance between cells (82% reduction) were seen in control cells 24 and 48 hours after scratch where the *MMP-9* gene was active. In cells treated with shRNA-MMP-9 those changes were 10.5% and 71%, and in doxycycline treated cells those changes were 29% and 63%. All results in HeLa cell migration, especially slowing down the migration of cells with *MMP-9* gene inhibited, were statistically significant. Observed changes of migration time considered with 23 hours of HeLa cells doubling time *in vitro*, and 19 hours half-life time confirmed effectiveness of inhibition of interfering RNA for the *MMP-9* gene [33]. A wound-healing assay is commonly used to analyze the time of cell migration and changes in that process after inhibition of particular genes responsible for tumor invasion. Researchers [41] investigated how inhibition of *MMP-9* by shRNA influences MMP-9 protein expression in glioblastoma multiforme cells. They observed a significant decrease (38% in comparison with 80% decrease of control cells) of migration potential 16 hours after scratch in shRNA-MMP-9 transfected cells. Changes in migration were also described by Franco et al. [9], where a statistically significant decrease of cell distance was observed in smooth muscle cells treated with doxycycline (104 μM) in comparison with control cells 48 hours after scratch. Antibiotic treated cells cover the scratch in 54% and control cells in 78%.

There is abundant evidence in the literature suggesting as clinically relevant inhibitors those that not only target catalytic domains of MMPs, but also disrupt MMP signaling (by blocking the hemopexin domain, or by

silencing *MMP* gene expression). For that reason small molecules, such as shRNAs, inhibiting MMP-9 mediated cancer cell migration and metastasis, as more specific and selective inhibitors, may perform better in the clinic than doxycycline hyclate. Our investigations confirm efficiency of the shRNA genetic construct used for *MMP-9* inhibition in HeLa cells *in vitro* at the protein level by zymography technique and wound-healing assay. Because of such a significant role of MMP-9 in cancer it is an interesting target point of potential gene therapy to control the level of extracellular matrix degradation by inhibiting expression of the mentioned gene. The successful results of presented studies encourage further research in this direction.

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